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Functional role of a conserved aspartic acid residue in the motor of the Na^+-driven flagellum from *Vibrio cholerae*

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ABSTRACT

The flagellar motor consists of a rotor and a stator and couples the flux of cations (H^+ or Na^+) to the generation of the torque necessary to drive flagellum rotation. The inner membrane proteins PomA and PomB are stator components of the Na^+-driven flagellar motor from *Vibrio cholerae*. Affinity-tagged variants of PomA and PomB were co-expressed in trans in the non-motile *V. cholerae pomAB* deletion strain to study the role of the conserved D23 in the transmembrane helix of PomB. At pH 9, the D23E variant restored motility to 100% of that observed with wild type PomB, whereas the D23N variant resulted in a non-motile phenotype, indicating that a carboxylic group at position 23 in PomB is important for flagellum rotation. Motility tests at decreasing pH revealed a pronounced decline of flagellar function with a motor complex containing the PomB-D23E variant. It is suggested that the protonation state of the glutamate residue at position 23 determines the performance of the flagellar motor by altering the affinity of Na^+ to PomB. The conserved aspartate residue in the transmembrane helix of PomB and its H^+-dependent homologs might act as a ligand for the coupling cation in the flagellar motor.

1. Introduction

Many bacteria are able to sense and swim by means of a flagellum which requires the products of approximately 50 genes for detecting and processing sensory cues, and for assembly and operation of the flagellar complex [1]. The flagellum of Gram-negative bacteria consists of the flagellar filament, a prolonged external spiral protein filament composed of flagellin, and the flagellar motor driving the rotation of the flagellar filament. The flagellar motor itself consists of a rotary and a statory part. The rotary part, also called basal body, comprises the L-, P-, MS- and C-ring and is similar to type-III secretion systems [2,3]. The statory part of the flagellar motor consists of 10–12 force generating units which are arranged in a circular array around the rotor [4,5]. In the H^+-driven *Escherichia coli* motor, each force generating unit is composed of two proteins designated MotA and MotB which form a $MotA_4B_2$ complex [4,6,7]. The $MotA_4B_2$ complex was demonstrated to function as H^+ channel [8,9], and, based on cross-linking data, a structural model for the $MotA_4B_2$ complex was proposed which includes a H^+-channel reaching from the periplasm to the strictly conserved Asp32 residue in the transmembrane helix of MotB [10]. The rotation of the MS-ring/FliG complex is believed to be driven by successive conformational changes in the $MotA_4B_2$ complex which are coupled to H^+ conduction [8–11]. Unproductive proton leakage through the flagellar motor was proposed to be prevented by a periplasmic region near the transmembrane helix of MotB [9,12].

In addition to H^+-dependent flagellar complexes, other systems exist which exclusively rely on sodium ions [13–15], or use either H^+ or Na^+ as coupling cations [16]. The inner membrane proteins PomA and PomB of the Na^+-dependent flagellar stator are homologs of MotA and MotB, respectively, whereas MotX and MotY are unique for Na^+-dependent motors and are localized at the periplasmic aspect of the outer membrane [17–19]. PomA, PomB, MotX, and MotY are required for torque generation by the Na^+-driven motors of *V. alginolyticus* and *V. parahaemolyticus* [20–23]. In *Vibrio cholerae*, genes encoding for PomA, PomB and other essential components of the Na^+-driven polar flagellar motor were identified [24,25] and the *V. cholerae* flagellum was shown to be driven by the sodium motive force [24]. PomA consists of four transmembrane helices with a large cytoplasmic loop between helices II and III, whereas PomB comprises only one transmembrane helix and a large periplasmic part including a peptidoglycan-binding motif, anchoring the stator to the cell wall (Fig. 1) [23,26]. A complex consisting of PomA and PomB exhibited Na^+ transport activity [27] and was shown to be composed of two PomA homodimers and one PomB homodimer [28]. In the H^+-driven flagellar motor from *E. coli*, the conserved Asp32 in the transmembrane region of MotB is required for motor function, and its protonation/deprotonation is proposed to cause conformational changes of the stator which drive flagellar rotation [29]. Consistent

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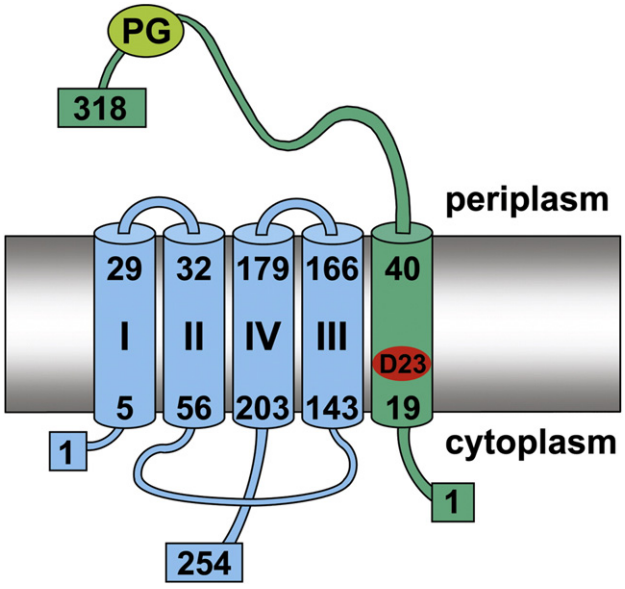

Fig. 1. Putative topology of the PomA and PomB stator components. PomA (blue) has a calculated mass of 27.4 kDa, PomB (green) of 35.4 kDa. According to a topology prediction using the program PSIPREP [51], PomA has four predicted transmembrane helices (I–IV). For *V. alginolyticus*, the third helix of PomA was shown to be in close proximity to the transmembrane helix of PomB [30]. The N-termini of PomA and PomB are located in the cytoplasm [52,53]. D23 is the only charged amino acid residue in the single transmembrane helix of PomB which is strictly conserved. PG: peptidoglycan-binding motif.

with such a model is the finding that mutations which neutralize the charge of Asp32 (e.g., replacement by Asn or Ala) alter the conformation of MotA in an assay based on protease susceptibility [11]. However, direct evidence for the transient protonation of Asp32 during torque generation has not been presented yet. In the Na^+-driven motor from *V. alginolyticus*, replacement of the corresponding Asp24 in PomB with Cys or Asn resulted in a non-motile phenotype [30,31].

Here we study the function of the corresponding aspartate residue (D23) in PomB of the Na^+-driven flagellar motor from *V. cholerae*. By analysing the dependence of motility on external pH, we show that the D23E variant of PomB confers full motility to the *V. cholerae* cells under alkaline but not under acidic conditions. The results are in accord with the hypothesis that protonation of the carboxylic group at position 23 of PomB impairs flagellar function, most likely by diminishing its affinity for Na^+. It is proposed that D23 in the transmembrane helix of PomB acts as a ligand for the coupling cation in the flagellar motor.

2. Materials and methods

2.1. Chemicals and bacterial strains

If not stated otherwise, chemicals were purchased from Sigma-Aldrich Chemie GmbH, Switzerland. Oligonucleotides were custom-synthesized by Microsynth AG, Switzerland. Yeast extract, tryptone and bacto agar were purchased from Becton Dickinson, USA. The *V. cholerae* strains used in this study were O395-N1 (the reference strain [Δ*ctxA* Str^r]) [32] and O395-N1 Δ*pomAB* (Δ*ctxA* Str^r Δ*pomAB*) [25]. For standard cloning procedures, *E. coli* strain DH5α served as host [33].

2.2. Cloning of V. cholerae pomAB

Standard procedures were carried out according to established protocols [33]. *V. cholerae* O395-N1 was grown on LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) in the presence of 50 μg/ml streptomycin. The *pomAB* genes were amplified by PCR (oligonucleotides VcmotAV and VcmotBR-I, Table S1) from genomic DNA [34] and blunt-end ligated to the EcoRV restricted vector pBluescript II SK(+) (Stratagene, USA), yielding plasmid pSK1805. The identity of the gene products of the cloned *pomAB* genes with the corresponding products of the *pomAB* genes of *V. cholerae* strain O395 (NC_009456; NC_009457) was confirmed by sequence analysis (oligonucleotides T3, T7, Vca307V, Vca609V, Vcb131V, Vcb437V; Table S1), and the obtained nucleotide sequence for *V. cholerae pomAB* from strain O395-N1 was submitted to the EMBL/GenBank/DDBJ database (AJ810450; AJ810451).

2.3. Construction of pAB, pAB-D23E and pAB-D23N

The *pomAB* genes were amplified from plasmid pSK1805 (oligonucleotides VcmotAV and VcmotBR-II) and inserted into vector pET224-Streptag (NdeI, XhoI), yielding plasmid pET18ABS (Table 1). Vector pET224-Streptag [35] is a derivative of pET24b (Novagen, USA) conferring a C-terminal Strep-tag [36] to a target protein. Vector pISC-H was obtained by introduction of the multiple cloning site of pISC-2 [37] into plasmid pEC422 [38] via restriction sites NdeI and EcoRI. No multi-copy side effects were reported with vector pEC422 used for the expression of membrane-bound subunits of the cytochrome *c* maturation system from *E. coli* [38]. Vector pISC-H adds an N-terminal His_6-tag to a target protein. Co-expression of *pomAB* in *V. cholerae* was achieved by subcloning the *pomA* and *pomB* genes from pET18ABS into vector pISC-H, yielding plasmid pAB encoding for PomA with an N-terminal His_6-tag and PomB with a C-terminal Strep-tag under the control of an arabinose-inducible promoter. In detail, the NdeI/Acc65I vector fragment of pISC-H was ligated to the *pomAB* genes amplified from plasmid pET18ABS using oligonucleotides Vcb980R-Acc65I and VcmotAV-II (Table S1) and restricted with NdeI/Acc65I. The *pomB* point mutations D23E and D23N were introduced into plasmid pAB by performing a two-step PCR procedure [39] using one pair of complementary oligonucleotides containing the codon for the desired mutation and two flanking oligonucleotides (VcmotAV-II and VcmotBR-II) whose sequence was derived from wild type (Table S1). Plasmid pET18ABS served as template. Restriction of the resulting 1761 bp *pomAB* PCR products with NdeI and XhoI and insertion into the NdeI/XhoI vector fragment of pAB yielded pAB-D23E and pAB-D23N (Table 1). The introduced mutations were confirmed by sequencing.

2.4. Transformation of V. cholerae

Electrocompetent cells of *V. cholerae* (O395-N1 and O395-N1 Δ*pomAB*) were prepared as described [40] and, immediately before an

Table 1
Plasmids.

Plasmid	Relevant characteristics	Reference
pET24b	Kan^r	Novagen, USA
pET224-Streptag	Kan^r	[35]
pBluescript II SK (+)	Ap^r	Stratagene, USA
pSK1805	Codes for *V. cholerae pomAB*; Ap^r	This study
pET18ABS	Codes for *V. cholerae pomAB*; PomB with C-terminal Strep-tag; Kan^r	This study
pISC-H	Confers His_6-tag to the N-terminus of a target protein; Ap^r	This study
pAB	Codes for His_6-PomA and PomB-Strep fusion proteins; Ap^r	This study
pAB-D23E	Codes for His_6-PomA and D23E variant of PomB-Strep; Ap^r	This study
pAB-D23N	Codes for His_6-PomA and D23N variant of PomB-Strep; Ap^r	This study

Ap^r, ampicillin resistance; Kan^r, kanamycin resistance.

electrical field was applied, 5 μl of salt-free plasmid DNA (50–300 ng/μl) was added. The mixture was transferred to a 2 mm cuvette (BioRad) and electroporated using a BioRad Gene Pulser with Pulse Controller (Parameter settings: 25 μF, 1.8 kV, 200 Ω; resulting time constants: 4.2–4.6). After addition of 500 μl LB medium, the cells were incubated for 1 h at 37 °C, plated out on LB agar (with 50 μg/ml streptomycin, 200 μg/ml ampicillin) and incubated for 14 h at 37 °C.

2.5. Motility assay

Transformants of *V. cholerae* (O395-N1 or O395-N1 Δ*pomAB*) with pAB, pAB-D23E, pAB-D23N or pISC-H, respectively, were grown overnight at 37 °C on LB agar plates (with 50 μg/ml streptomycin, 200 μg/ml ampicillin) supplemented with 5 mM arabinose. Single colonies were stabbed into motility agar plates (LB with 0.3% bacto agar, 10 mM KP_i pH 7.0, 7.5, 8.0, 8.5 or 9.0, 100 μg/ml ampicillin, 5 mM arabinose) using a pointed, round toothpick [41]. If not stated otherwise, the diameters of the motility rings were determined after 20 h at 30 °C.

2.6. Determination of swimming speed

V. cholerae Δ*pomAB* containing plasmid pAB or pAB-D23E, respectively, was grown overnight at 37 °C in 3 ml LB (with 50 μg/ml streptomycin, 200 μg/ml ampicillin). One ml LB (with 50 μg/ml streptomycin, 200 μg/ml ampicillin, 50 mM KP_i pH 7.0, 7.5, 8.0, 8.5 or 9.0) was inoculated with 2% of the overnight culture and cells were grown at 37 °C. At $OD_{600} = 0.7$, *pomAB* expression was induced by adding 15 mM arabinose. To visualize the cells by fluorescence, 250 nM MitoTracker Red CMXRos (Invitrogen, USA) was added from a 0.1 mM stock solution in dimethylsulfoxide. To suppress changes in swimming direction, 50 mM L-serine was included in the medium [42]. One hour after induction, the culture was diluted 1:250 in LB with 50 mM KP_i pH 7.0, 7.5, 8.0, 8.5 or 9.0, supplemented with 50 mM L-serine, 15 mM arabinose and 200 μg/ml ampicillin. The cultures were immediately inspected by microscopy at 25 °C using an inverted fluorescence microscope (Leica DM6000-LX; Leica Microsystems GmbH, Germany) equipped with an EM-CCD fluorescence camera (Hamamatsu C9100-13; Hamamatsu Photonics, Japan), a 20× multi immersion objective and a regular TexasRed fluorescence filter. 100 μl of the diluted culture was pipetted into a channel of an Ibidi 1 μ slide VI (Integrated BioDiagnostics, Germany), and after focussing in the *z*-axis to the middle between the base and the top of the channel, about 100 series of time sequences were taken (in overlapping mode, 16 frames at 40 ms each). Using image processing (Imaris; Bitplane AG, Switzerland) bacteria were recognized and detected by a spot detection algorithm. An average velocity for each individual bacterium was calculated by a tracking algorithm only if the bacterium could be constantly followed for at least 320 ms. Tracks of bacteria displaying average speeds between 4 and 30 μm s^{-1} were added (representing 100%), and the percentages of motile cells displaying speeds between 18 and 30 μm s^{-1} were cumulated. In a single experiment, the number of individual tracks analysed in the speed range of 4–30 μm s^{-1} was >1000 at each pH. Acquisition and image processing settings were kept constant.

2.7. Expression of PomA and of PomB variants

V. cholerae Δ*pomAB*, transformed with plasmid pAB, pAB-D23E or pAB-D23N, respectively, was grown overnight at 37 °C in 10 ml LB (with 50 μg/ml streptomycin, 200 μg/ml ampicillin). Cells were collected by centrifugation and resuspended in LB to a final OD_{600} of 5.0. One ml of each cell suspension was used to inoculate 100 ml LB (with 50 μg/ml streptomycin, 200 μg/ml ampicillin, 50 mM KP_i pH 7.0, 7.5, 8.0, 8.5 or 9.0). The cultures were grown at 37 °C, and at $OD_{600} = 0.7$, 15 mM arabinose was added to induce *pomAB* expression. Aliquots of the cultures (1 ml) were removed immediately before and 3 h after induction for Western blot analysis. The protein content of *V. cholerae* cell suspensions was estimated from the optical density, assuming that 1 U of absorbance at 600 nm corresponds to 0.33 g total dry weight per liter [43] and that 55% of the total dry weight represents protein [44]. Cells were resuspended in loading buffer (50 mM Tris–HCl pH 6.8, 1% SDS, 10% glycerol, 100 mM 1,4-dithiothreithol, bromophenol blue) to a protein concentration of 1 mg/ml.

From cells grown as described above, membranes were prepared as follows. Cells were resuspended in 3 ml extraction buffer (50 mM Tris–HCl pH 8.0, 300 mM NaCl, 0.5 mM $MgCl_2$). Few crystals of DNase I were added, and 0.2 mM diisopropyl fluorophosphate was added prior to cell rupture by three passages through a French Pressure Mini cell at 7 MPa. Unbroken cells were removed by centrifugation (10,000 ×*g*, 30 min, 4 °C) and the membranes were separated from the supernatant by ultracentrifugation (200,000 ×*g*, 1 h, 4 °C). The membranes were resuspended in extraction buffer, and their protein content was determined by the bicinchoninic acid method using the reagent from Pierce [45]. Prior to SDS-PAGE, the membranes were diluted in loading buffer to a protein concentration of 1 mg/ml.

2.8. SDS-PAGE and Western blots

SDS-PAGE was performed according to Schägger and von Jagow [46] with 12% acrylamide/bisacrylamide (37.5:1.0; National Diagnostics, USA). Prestained broad range protein marker (New England Biolabs, USA) served as molecular weight standard. Electroblotting of proteins from SDS-PAGE to a nitrocellulose membrane was performed according to the semi-dry protocol described by the manufacturer (BioRad, USA). After protein transfer, the nitrocellulose membrane was washed twice for 10 min with TBS (20 mM Tris–HCl pH 7.5, 500 mM NaCl) and incubated in 3% BSA (in TBS) for 2 h at RT. Three washing steps (10 min) with TTBS (TBS with 0.05% Tween-20) were followed by co-incubation with Ni-NTA-HRP conjugate (Qiagen AG, Switzerland; 1:1000 in TBS) and Strep-Tactin-HRP conjugate (IBA GmbH, Germany; 1:1000 in TBS) for 1 h at RT. After three washing steps (10 min) in TTBS, immunostaining was carried out in 20 ml TBS containing 130 μl 3% 4-chloro-1-naphthol (in methanol) and 20 μl 30% H_2O_2. The chromogenic reaction was stopped by rinsing the membrane with ddH_2O. The developed Western blots were immediately photographed (Syngene Gene Genius, Synoptics Ltd., Great Britain), and the volumes of PomB bands were determined using the Quantity One software (BioRad, USA).

3. Results

3.1. Production of His-PomA and PomB-Strep in V. cholerae

To study the function of the stator component PomB by means of site-directed mutagenesis, we constructed plasmid pAB containing *pomA* and *pomB* under the control of an arabinose-inducible promoter (Table 1). We chose to produce PomA together with PomB to ensure that the assembly or insertion of force generating units containing PomA and PomB was not limiting in complementation studies with the non-motile *pomAB* deletion strain of *V. cholerae*. PomA expressed from pAB contains a His-tag at its N-terminus, PomB a Strep-tag at its C-terminus. In the following, we will refer to the fusion proteins as PomA and wild type PomB. Plasmids pAB-D23E and pAB-D23N encode for the D23E and D23N variants of PomB. Production of PomA and PomB in *V. cholerae* Δ*pomAB* was confirmed by immunostaining of Western blots. Prior to the addition of arabinose, none of the cultures produced PomA or PomB at detectable levels, but co-expression of the three PomB variants with PomA was observed upon induction (wild type, D23E and D23N) (Fig. S1). The calculated molecular masses of PomA and PomB (including tags) are 29.6 and 36.7 kDa, respectively.

The apparent mass of PomB observed on SDS-PAGE was consistent with the calculated mass, whereas a smaller apparent mass due to faster migration was observed with the very hydrophobic PomA.

We now asked if the insertion of a mutation at position 23 had an effect on the expression level of PomB and determined the relative amount of the D23E and D23N PomB variants in comparison to wild type PomB using *V. cholerae* Δ*pomAB* as expression host. Western blot analysis was followed by quantification of the signal intensity arising from PomB. Using serial dilutions of whole cells containing wild type PomB, it was verified that, in the applied concentration range, the amount of PomB subjected to Western blot analysis was proportional to the observed signal intensity. Compared to wild type PomB (100%), the relative concentrations of mutant PomB in Δ*pomAB* cells co-expressing PomA were 93% for PomB-D23E and 117% for PomB-D23N (Fig. 2A) at pH 8. The analysis was repeated using washed membranes obtained from *V. cholerae* Δ*pomAB* cells expressing PomB wild type and mutant variants at pH 8. Here, the relative amount of PomB (D23E or D23N) compared to wild type PomB (100%) was 67% or 91%, respectively (Fig. 2B). These results demonstrated that introduction of the D23E and D23N mutations in PomB did not alter the amount of PomB present in the cells compared to wild type PomB. We also checked if the expression level of wild type or the D23E variant of PomB was influenced by the pH of the growth medium. In the range from pH 7 to 9, the amount of PomB and PomB-D23E produced by *V. cholerae* cells remained constant (Fig. 2C). Overexpression of PomB variants (wild type, D23E and D23N) had no effect on growth rates or cell yields of the *V. cholerae* expression host under the specified growth conditions (not shown). Taken together, these findings allowed studying the role of aspartate 23 in PomB on flagellar function by an in vivo approach, as outlined below.

3.2. Complementation of the motility defect of a *V. cholerae pomAB* deletion strain

The deletion of *pomAB* in *V. cholerae* results in a flagellated but non-motile phenotype [25]. We asked if motility was restored in the presence of plasmid pAB encoding for the PomA and PomB fusion proteins, and tested transformants of the *pomAB* deletion strain on motility plates containing 170 mM Na^+ which is a saturating Na^+-concentration for the Na^+-driven polar flagellar motor of *V. cholerae* [24]. A few hours of incubation at 30 °C led to the appearance of a small motility ring which was well defined by its outer brim. After 20 h, the diameter of the ring was measured and compared to that formed by the *V. cholerae* reference strain transformed with the empty vector pISC-H (representing 100%) (Fig. 3A). The obtained average value for complementation of motility was $99.3 \pm 5.0\%$ ($n = 8$). It is concluded that the fusion of a His-tag to the N-terminus of PomA, and of a Strep-tag to the C-terminus of PomB, did not interfere with assembly or membrane insertion of the flagellar motor. No motility was observed with the *V. cholerae pomAB* deletion strain transformed with the control vector (Fig. 3A).

In *E. coli* MotB, an aspartate at position 32 (equivalent to position 23 in *V. cholerae* PomB) was demonstrated to be essential for motor function [29]. Of several different substitutions studied at this position, only MotB-D32E retained some function (less than 10% of the swarming rate observed with wild type). Replacement of Asp32 with Asn resulted in a non-motile phenotype [29]. In *Salmonella typhimurium*, the MotB-D33E mutant (corresponding to Asp23 in *V. cholerae*) exhibited 20% of the motility of the wild type [47]. Rotation of the flagellar complex from *E. coli* is driven by the proton motive force. To study the impact of the corresponding amino acid substitutions on the function of the Na^+-driven flagellum, we determined the motility of the *V. cholerae* reference and Δ*pomAB* strains containing PomA together with wild type PomB, the PomB-D23N or PomB-D23E variants (Fig. 3B, C). At pH 8, the presence of wild type PomB and the PomB-D23E variant in the Δ*pomAB* strain resulted in a functional flagellar complex ($100.0 \pm 4.3\%$ and $83.6 \pm 11.3\%$ of the motility observed with the *V. cholerae* reference strain) (Fig. 3B). The *V. cholerae* reference and Δ*pomAB* strains transformed with pAB

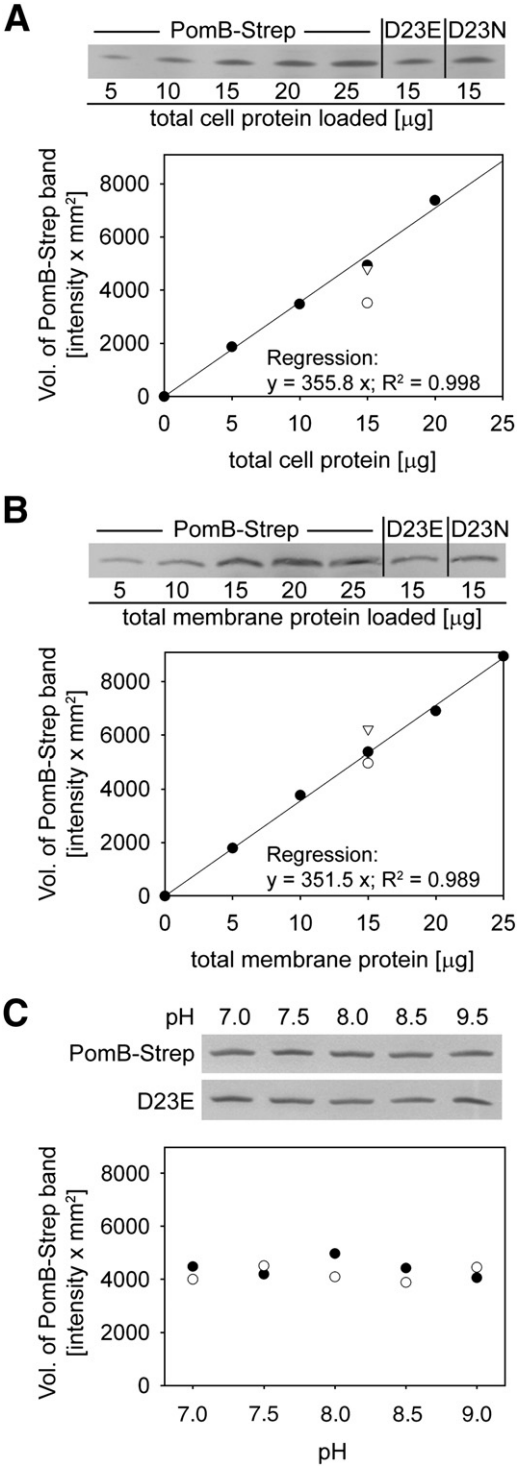

Fig. 2. Expression levels of wild type PomB and its D23E and D23N variants. (A) Aliquots of *V. cholerae* Δ*pomAB* cells (5–25 μg protein) grown at pH 8 expressing wild type PomB (closed circles), PomB-D23E (open circles) or D23N (triangles) were subjected to Western blot analysis followed by immunostaining (upper panel) and quantification of the streptavidin tag of PomB (lower panel). Within the applied protein concentration range, the signal intensity arising from wild type PomB increased linearly with the amount of protein loaded. (B) Same as in (A), but with membranes. (C) Upper panel, Western blot of *V. cholerae* Δ*pomAB* cells (15 μg protein) grown at pH 7, 7.5, 8, 8.5 or 9 expressing wild type PomB (PomB-Strep) or its D23E variant (D23E). Lower panel, quantification of the streptavidin tag of wild type PomB (closed circles) or PomB-D23E (open circles).

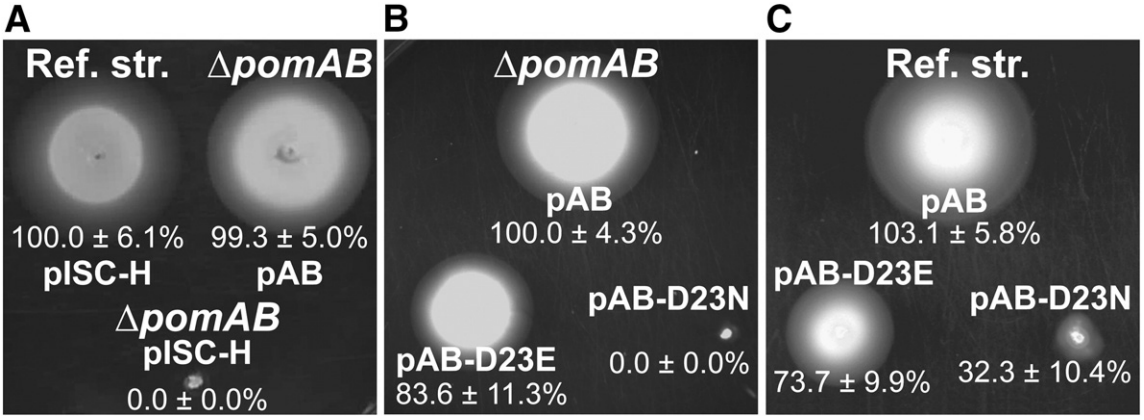

Fig. 3. Motility of the *V. cholerae* reference and *pomAB* deletion strains at pH 8. (A) Reference strain (Ref. str.) transformed with the control vector (pISC-H), and *V. cholerae* lacking PomAB (Δ*pomAB*) transformed with pAB coding for His-PomA and PomB-Strep or with pISC-H. The diameter of the motility ring from *V. cholerae* Δ*pomAB* transformed with pAB represents the reference value (100%), and the motilities of the other transformants were quantified as percentages of that. (B) Motility of *V. cholerae* Δ*pomAB* producing PomA together with wild type PomB, PomB-D23E or PomB-D23N. (C) Motility of *V. cholerae* reference strain producing PomA together with wild type PomB, PomB-D23E or PomB-D23N. Mean values from at least four independent experiments are presented.

exhibited very similar motilities (Fig. 3B, C). As observed with the proton-dependent MotB stator component, changing Asp23 of PomB to Asn resulted in a non-motile phenotype (Fig. 3B). Importantly, the D23N substitution of PomB exerted a dominant negative effect on motility in the reference *V. cholerae* strain which contains a chromosomal copy of the *pomB* gene (Fig. 3C). This further confirmed that the PomA and PomB fusion proteins studied here were assembled into the flagellar complex in vivo. The function of the Na^+-driven motor depended on a carboxylic residue at the conserved position D23 in the transmembrane helix of PomB. At pH 8, replacement of D23 with a glutamate residue resulted in almost full functional recovery of the Na^+-dependent motor. This is in marked contrast to H^+-dependent motors, where less than 10% of wild type motility was observed in the presence of the MotB-D32E variant [29]. We further asked whether the external proton concentration had an effect on the function of the flagellar motor, and determined the motility of the *V. cholerae* reference and *pomAB* deletion strains transformed with pAB or pAB-D23N at pH 7, 8 and 9. Importantly, the genetic background did not influence the motility in the presence of wild type PomB at any pH tested. The dominant negative effect of pAB-D23N on motility of the *V. cholerae* reference strain at pH 8 was not diminished by shifting the pH to the more acidic or more alkaline range (not shown).

3.3. A flagellar complex containing PomB-D23E exhibits altered pH profile of motility

An advantage studying the Na^+-coupled rather than the H^+-coupled flagellum is that possible effects of protonation or deprotonation can be studied by varying the pH without changing the concentration of the coupling cation (Na^+). We asked whether the function of the flagellar motor was influenced by the external pH, and determined the motility of *V. cholerae* containing wild type PomB or the PomB variant with the glutamate replacing the aspartate at position 23 (PomB-D23E) under a saturating Na^+ concentration (170 mM) at different external proton concentrations. We first inspected the motility of the cells in soft agar plates 7.5 h after inoculation. An impairment of flagellar function introduced by the PomB-D23E variant was evident when motility was analysed at pH 8 or lower. At pH 7, after an incubation period of 7.5 h at 37 °C, the diameter of the motility ring formed by cells with a flagellum containing the PomB-D23E variant was significantly smaller than that formed by cells with wild type PomB (0.35 ± 0.05 cm versus 0.80 ± 0.01 cm) (Fig. 4A). At pH 7.5 and 8, motility of *V. cholerae* using the D23E mutant of the PomB stator component was also severely reduced compared to cells using wild type PomB. In contrast, cells containing a flagellum with PomB-D23E exhibited high motilities (0.7 ± 0.01 cm or 0.65 ± 0.05 cm) at pH 8.5 and 9 which were indistinguishable from that of wild type cells (0.75 ± 0.05 cm or 0.7 ± 0.01 cm) (Fig. 4A). The flagellar complex of *V. cholerae* containing wild type PomB worked with an essentially constant, high efficiency over the whole range from pH 7 to 9. Very similar results were obtained when motility was checked 9.5 h after inoculation (data not shown).

We then investigated if the external pH had an effect on motility of *V. cholerae* containing wild type or D23E-PomB in liquid medium. Cells were labelled with a fluorescent dye, and the swimming speeds of individual bacteria were determined. At pH 9, 8.5 and 8, the fraction of cells exhibiting swimming speeds between 18 and 30 μm s^{-1} was identical in the presence of wild type PomB or the D23E variant (Fig. 4B). In the presence of wild type PomB, increasing the external proton concentration (pH 7.5 and 7) resulted in an increase of the fraction of

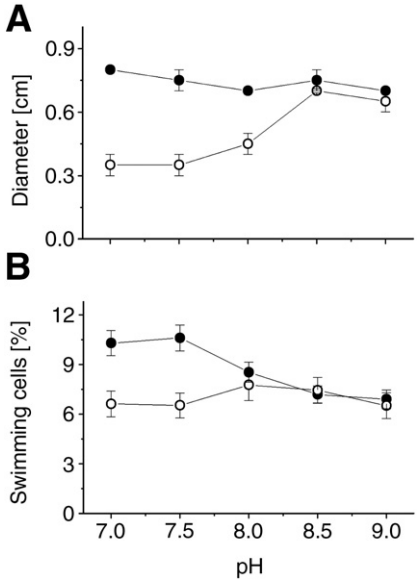

Fig. 4. Influence of external pH on motility and swimming speed of the *V. cholerae pomAB* deletion strain producing PomB or PomB-D23E. (A) Motility in soft agar plates. The diameters of the motility rings of *V. cholerae* Δ*pomAB* producing wild type PomB (closed circles) or PomB-D23E (open circles) were determined after 7.5 h at 37 °C. Mean values from two independent experiments are presented. Vertical bars indicate standard errors. (B) Swimming speeds in liquid culture. Percentage of motile cells (18–30 μm s^{-1}) compared to the total of cells exhibiting swimming speeds between 4 and 30 μm s^{-1} (100%).

cells exhibiting high motility. In contrast, the fraction of motile cells containing PomB-D23E was not influenced by external pH. The results indicate that at more acidic external pH, wild type PomB confers higher motility than the D23E variant of the protein. The data are in accord with the increased swarming ability of *V. cholerae* containing wild type PomB compared to PomB-D23E observed under acidic conditions in motility plates (Fig. 4A).

We conclude that the external pH of the medium, representing the proton concentration in the periplasm [48], has an influence on rotation of the polar flagellum of *V. cholerae*. At elevated external proton concentrations, the flagellar complex containing the PomB-D23E variant showed decreased functional performance compared to the wild type complex.

4. Discussion

The bacterial flagellum is driven by a motor that converts the electrochemical gradient of the coupling cation (H^+ or Na^+) into mechanical torque. Rotational force is proposed to be generated via interactions of the stator elements with the rotor elements of the motor. In the flagellar motor, MotA and MotB, or the homologous subunits PomA and PomB, form the stator complex and function in H^+ (MotA, MotB) or Na^+ transport (PomA, PomB). The molecular details of the interaction of the coupling cations with the stator complexes, and their translocation pathways through these inner membrane proteins, are unknown. Asp32 located in the single transmembrane helix of *E. coli* MotB [29], and the corresponding Asp23 in *V. cholerae* PomB (this study), were shown to be required for motor function. We envisage the flagellar motor as a machine performing many different, distinct reaction steps. In the presence of wild type PomB, some of these steps may depend on the external (periplasmic) proton concentration. By replacing aspartate 23 in PomB with glutamate, the susceptibility of the machine towards pH is more pronounced; flagellar activity reaches wild type levels at pH 9, but, compared to the wild type motor, overall performance drops significantly at increased periplasmic proton concentrations. This is an important observation since other parameters which might influence flagellar rotation, like the transmembrane gradients for sodium ions, and the transmembrane voltage, are not altered; for each external proton concentration studied, we can assume that the electrochemical Na^+ gradient driving the flagellum is identical in the *V. cholerae pomAB* deletion strain containing wild type PomB or its D23E variant.

The question is whether D23 in PomB, or D32 in MotB, play an indirect functional role, for example by stabilizing a distinct conformation of the motor complex, or directly participate in conducting Na^+ or H^+ through the membrane. Our finding that, at pH 8.5 and 9, a motor containing the PomB-D23E variant confers full motility, and the fact that the aspartate and glutamate residues only differ in side chain length by one CH_2 unit, are strong arguments against large conformational changes introduced by mutations at position 23 as indirect cause for impairment of motor function. For the H^+-driven flagellum from *S. typhimurium*, Che et al. showed that the D33E variant of MotB (corresponding to PomB-D23E from *V. cholerae*) exhibited only 20% of the motility of the wild type [47]. By analysing the torque–speed relationship of the D33E motor and its functional revertants carrying missense mutations in MotAB, the authors proposed that introducing an aspartate at position 33 not only reduces H^+ conductivity but also indirectly diminishes flagellar performance by interfering with the torque generation step. Our results obtained with the related, Na^+-dependent flagellum do not support the idea of an indirect role of Asp23 as both wild type PomB and the D23E variant exhibited full functional performance under alkaline conditions and saturating coupling cation concentration. We therefore favor a direct effect of the D23E substitution on flagellar performance and speculate that the protonation state of the glutamate residue at position 23 is determined by the outer pH. The Asp-Glu substitution might cause a diminished affinity of the PomB binding site for Na^+, allowing the carboxylic group of the glutamate to be protonated at elevated external H^+ concentrations. The pK_a of D and E in aqueous solution is essentially identical (pK_a (D) = 3.9, pK_a (E) = 4.1) [49] but shifts towards the alkaline range in hydrophobic regions of membrane proteins where it is strongly influenced by the local environment [50]. Therefore, the dissociation constants for D23 and E23 in PomB may be different. We hypothesise that in the wild type stator complex, the carboxylic group of D23 is deprotonated and acts as a ligand for Na^+ under all pH conditions tested. Replacing aspartate with glutamate might result in a slight distortion of the coupling cation binding site, allowing protonation of the carboxylic group of D23 under more acidic conditions. Na^+ binding to a motor complex containing PomB-D23E would still be possible under alkaline conditions when the external proton concentration is low, as observed in our experiments. Our speculation of protons and sodium ions competing for binding to D23 in PomB is further supported by the observation that insertion of an asparagine at position 23, which mimics Asp23 in its protonated state, blocked motility completely. Another possible explanation would be that the structure of the PomA/B complex is changed by the outer pH, making the channel leading to E23 more sensitive for pH changes than the wild type channel. The flagellar complex containing the PomB-D23E variant described here represents a model system to address this problem in future studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.05.015.

References

[1] R.M. Macnab, Genetics and biogenesis of bacterial flagella, Annu. Rev. Genet. 26 (1992) 131–158.
[2] T. Minamino, K. Namba, Self-assembly and type III protein export of the bacterial flagellum, J. Mol. Microbiol. Biotechnol. 7 (2004) 5–17.
[3] Y. Sowa, R.M. Berry, Bacterial flagellar motor, Q. Rev. Biophys. 41 (2008) 103–132.
[4] S. Khan, M. Dapice, T.S. Reese, Effects of *mot* gene expression on the structure of the flagellar motor, J. Mol. Biol. 202 (1988) 575–584.
[5] S.W. Reid, M.C. Leake, J.H. Chandler, C.J. Lo, J.P. Armitage, R.M. Berry, The maximum number of torque-generating units in the flagellar motor of *Escherichia coli* is at least 11, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 8066–8071.
[6] S.M. Block, H.C. Berg, Successive incorporation of force-generating units in the bacterial rotary motor, Nature 309 (1984) 470–472.
[7] D.F. Blair, H.C. Berg, Restoration of torque in defective flagellar motors, Science 242 (1988) 1678–1681.
[8] D.F. Blair, H.C. Berg, The MotA protein of *E. coli* is a proton-conducting component of the flagellar motor, Cell 60 (1990) 439–449.
[9] B. Stolz, H.C. Berg, Evidence for interactions between MotA and MotB, torque-generating elements of the flagellar motor of *Escherichia coli*, J. Bacteriol. 173 (1991) 7033–7037.
[10] E.A. Kim, M. Price-Carter, W.C. Carlquist, D.F. Blair, Membrane segment organization in the stator complex of the flagellar motor: implications for proton flow and proton-induced conformational change, Biochemistry 47 (2008) 11332–11339.
[11] S. Kojima, D.F. Blair, Conformational change in the stator of the bacterial flagellar motor, Biochemistry 40 (2001) 13041–13050.
[12] E.R. Hosking, C. Vogt, E.P. Bakker, M.D. Manson, The *Escherichia coli* MotAB proton channel unplugged, J. Mol. Biol. 364 (2006) 921–937.
[13] M. Ito, D.B. Hicks, T.M. Henkin, A.A. Guffanti, B.D. Powers, L. Zvi, K. Uematsu, T.A. Krulwich, MotPS is the stator-force generator for motility of alkaliphilic *Bacillus*, and its homologue is a second functional Mot in *Bacillus subtilis*, Mol. Microbiol. 53 (2004) 1035–1049.

[14] L.L. McCarter, Polar flagellar motility of the *Vibrionaceae*, Microbiol. Mol. Biol. Rev. 65 (2001) 445–462.
[15] T. Yorimitsu, M. Homma, Na^+-driven flagellar motor of *Vibrio*, Biochim. Biophys. Acta 1505 (2001) 82–93.
[16] N. Terahara, T.A. Krulwich, M. Ito, Mutations alter the sodium versus proton use of a *Bacillus clausii* flagellar motor and confer dual ion use on *Bacillus subtilis* motors, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 14359–14364.
[17] M. Okabe, T. Yakushi, M. Kojima, M. Homma, MotX and MotY, specific components of the sodium-driven flagellar motor, colocalize to the outer membrane in *Vibrio alginolyticus*, Mol. Microbiol. 46 (2002) 125–134.
[18] H. Terashima, H. Fukuoka, T. Yakushi, S. Kojima, M. Homma, The *Vibrio* motor proteins, MotX and MotY, are associated with the basal body of Na^+-driven flagella and required for stator formation, Mol. Microbiol. 62 (2006) 1170–1180.
[19] S. Kojima, A. Shinohara, H. Terashima, T. Yakushi, M. Sakuma, M. Homma, K. Namba, K. Imada, Insights into the stator assembly of the *Vibrio* flagellar motor from the crystal structure of MotY, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 7696–7701.
[20] L.L. McCarter, MotX, the channel component of the sodium-type flagellar motor, J. Bacteriol. 176 (1994) 5988–5998.
[21] L.L. McCarter, MotY, a component of the sodium-type flagellar motor, J. Bacteriol. 176 (1994) 4219–4225.
[22] I. Okunishi, I. Kawagishi, M. Homma, Cloning and characterization of *motY*, a gene coding for a component of the sodium-driven flagellar motor in *Vibrio alginolyticus*, J. Bacteriol. 178 (1996) 2409–2415.
[23] Y. Asai, S. Kojima, H. Kato, N. Nishioka, I. Kawagishi, M. Homma, Putative channel components for the fast-rotating sodium-driven flagellar motor of a marine bacterium, J. Bacteriol. 179 (1997) 5104–5110.
[24] S. Kojima, K. Yamamoto, I. Kawagishi, M. Homma, The polar flagellar motor of *Vibrio cholerae* is driven by an Na^+ motive force, J. Bacteriol. 181 (1999) 1927–1930.
[25] K.K. Gosink, C.C. Häse, Requirements for conversion of the Na^+-driven flagellar motor of *Vibrio cholerae* to the H^+-driven motor of *Escherichia coli*, J. Bacteriol. 182 (2000) 4234–4240.
[26] A. Roujeinikova, Crystal structure of the cell wall anchor domain of MotB, a stator component of the bacterial flagellar motor: implications for peptidoglycan recognition, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 10348–10353.
[27] K. Sato, M. Homma, Functional reconstitution of the Na^+-driven polar flagellar motor component of *Vibrio alginolyticus*, J. Biol. Chem. 275 (2000) 5718–5722.
[28] K. Sato, M. Homma, Multimeric structure of PomA, a component of the Na^+-driven polar flagellar motor of *Vibrio alginolyticus*, J. Biol. Chem. 275 (2000) 20223–20228.
[29] J. Zhou, L.L. Sharp, H.L. Tang, S.A. Lloyd, S. Billings, T.F. Braun, D.F. Blair, Function of protonatable residues in the flagellar motor of *Escherichia coli*: a critical role for Asp32 of MotB, J. Bacteriol. 180 (1998) 2729–2735.
[30] T. Yakushi, S. Maki, M. Homma, Interaction of PomB with the third transmembrane segment of PomA in the Na^+-driven polar flagellum of *Vibrio alginolyticus*, J. Bacteriol. 186 (2004) 5281–5291.
[31] H. Fukuoka, T. Wada, S. Kojima, A. Ishijima, M. Homma, Sodium-dependent dynamic assembly of membrane complexes in sodium-driven flagellar motors, Mol. Microbiol. 71 (2009) 825–835.
[32] J.J. Mekalanos, D.J. Swartz, G.D. Pearson, N. Harford, F. Groyne, M. de Wilde, Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development, Nature 306 (1983) 551–557.
[33] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor NY, 1989.
[34] F. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds.), Short Protocols in Molecular Biology, 3rd ed. Wiley, 1995.
[35] K. Schneider, P. Dimroth, M. Bott, Biosynthesis of the prosthetic group of citrate lyase, Biochemistry 39 (2000) 9438–9450.
[36] T.G. Schmidt, A. Skerra, The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins, Nat. Protoc. 2 (2007) 1528–1535.
[37] E. Arslan, H. Schulz, R. Zufferey, P. Künzler, L. Thöny-Meyer, Overproduction of the *Bradyrhizobium japonicum c*-type cytochrome subunits of the cbb_3 oxidase in *Escherichia coli*, Biochem. Biophys. Res. Commun. 251 (1998) 744–747.
[38] H. Schulz, R.A. Fabianek, E.C. Pellicioli, H. Hennecke, L. Thöny-Meyer, Heme transfer to the heme chaperone CcmE during cytochrome *c* maturation requires the CcmC protein, which may function independently of the ABC-transporter CcmAB, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 6462–6467.
[39] S. Barik, Site directed mutagenesis by double polymerase chain reaction: megaprimer method, in: B.A. White (Ed.), PCR protocols: current methods and applications, Humana Press, 1993, pp. 277–287.
[40] H. Marcus, J.M. Ketley, J.B. Kaper, R.K. Holmes, Effects of DNase production, plasmid size, and restriction barriers on transformation of *Vibrio cholerae* by electroporation and osmotic shock, FEMS Microbiol. Lett. 56 (1990) 149–154.
[41] C.L. Gardel, J.J. Mekalanos, Alterations in *Vibrio cholerae* motility phenotypes correlate with changes in virulence factor expression, Infect. Immun. 64 (1996) 2246–2255.
[42] S. Yoshida, S. Sugiyama, Y. Hojo, H. Tokuda, Y. Imae, Intracellular Na^+ kinetically interferes with the rotation of the Na^+-driven flagellar motors of *Vibrio alginolyticus*, J. Biol. Chem. 265 (1990) 20346–20350.
[43] S.A. Underwood, M.L. Buszko, K.T. Shanmugam, L.O. Ingram, Lack of protective osmolytes limits final cell density and volumetric productivity of ethanologenic *Escherichia coli* KO11 during xylose fermentation, Appl. Environ. Microbiol. 70 (2004) 2734–2740.
[44] F.C. Neidhardt, J.L. Ingraham, R. Curtiss III, E.C.C. Lin, K. Brooks Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, H.E. Umbarger, *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, 2nd ed. American Society for Microbiology, Washington, D.C., 1996.
[45] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[46] H. Schägger, G. von Jagow, Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, Anal. Biochem. 166 (1987) 368–379.
[47] Y.S. Che, S. Nakamura, S. Kojima, N. Kami-ike, K. Namba, T. Minamino, Suppressor analysis of the MotB(D33E) mutation to probe bacterial flagellar motor dynamics coupled with proton translocation, J. Bacteriol. 190 (2008) 6660–6667.
[48] E. Padan, The enlightening encounter between structure and function in the NhaA Na^+–H^+ antiporter, Trends Biochem. Sci. 33 (2008) 435–443.
[49] R.M.C. Dawson, D.C. Elliott, W.H. Elliott, K.M. Jones, Data for Biochemical Research, 2nd ed. Oxford University Press, 1969.
[50] C. Kluge, P. Dimroth, Kinetics of inactivation of the F_1F_0 ATPase of *Propionigenium modestum* by dicyclohexylcarbodiimide in relationship to H^+ and Na^+ concentration: probing the binding site for the coupling ions, Biochemistry 32 (1993) 10378–10386.
[51] L.J. McGuffin, K. Bryson, D.T. Jones, The PSIPRED protein structure prediction server, Bioinformatics 16 (2000) 404–405.
[52] S.Y. Chun, J.S. Parkinson, Bacterial motility: membrane topology of the *Escherichia coli* MotB protein, Science 239 (1988) 276–278.
[53] J. Zhou, R.T. Fazzio, D.F. Blair, Membrane topology of the MotA protein of *Escherichia coli*, J. Mol. Biol. 251 (1995) 237–242.